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DYNAMICS OF MULTIPLICATION OF THE VEE VIRUS IN TISSUE CULTURE CELLS

/Following is the translation of an article by F. I. Yershov and V. A. Vagzhanova, Institute of Virology imeni D. I. Ivanovskago, USSR Academy of Medical Sciences, Moscow, published in the Russian-language periodical Voprosy Virusologii (Problems of Virology), No 2, 1965, pages 176--180. It was submitted on 30 Oct 1963. Translation performed by Sp/7 Charles T. Ostertag Jr./

Among the viruses which cause the destruction and breakdown of cells, the viruses of the equine encephalomyelites occupy a particular place. They are highly pathogenic for man and the majority of animals and capable of affecting initial and transplanted tissue cultures /5, 11, 13, 25/. A study of the interrelationships of this group of viruses with cells is of great interest, however up until the present time this problem has hardly been dealt with in the literature.

The aim of the present work was a study of the regularities of multiplication of the virus of Venezuelan encephalomyelitis in tissue culture cells.

Materials and Methods

The strain of the Venezuelan equine encephalomyelitis (VEE) virus used by us had gone through 3 passages on mice and 17 subsequeht passages on a culture of chick embryo fibroblasts. The virus containing material was a cultural liquid, collected after 24 hours following infection and stored at 4°. The titer of the virus comprised $10^{-8.8}$--10^{-9} 1g CPD_{50}.

For the majority of the tests we used a culture of chick embryo fibroblasts, prepared according to the generally accepted method /1/; several tests were set up with HeLa and SOTs cells. For cultivation we used medium 199 with 10% bovine serum. The culture was infected following the formation of a monolayer. After a specific interval of time following introduction of the virus, samples of the cultural fluid were withdrawn and the infectious titer was determined, and also hemagglutinating and complement fixing antigens. At the same time we determined the cytopathological effect of the virus and set up the hemadsorption reaction.

Titration of the virus was carried out in tissue culture and in animals. By the intracerebral route, mice weighing 6--8 grams received 0.03 ml each of a 10x dilution of the virus. The same dose was used for the infection of the tissue cultures and embryos. The results of the titration were considered based on the cytopathological effect, the formation of plaques, or the death

of the animals. The value of the TCD_{50} was calculated by the method of Kerber /17/. The competence of titrating VEE on tissue culture was shown earlier in the works of Kissling /18/ and N. V. Kaverina /4/, who established the existence of a parallelism between the CPD of the virus and its virulence for animals.

For setting up the RGA /hemagglutination reaction/ we used the principles developed by Clarke and Casals in respect to arboviruses /8/: The cultural fluid under investigation was diluted in a borate buffer (pH 9.0), and the suspension of erythrocytes was prepared in phosphate buffers with various pH values. The results were considered after a 45--60 minute contact of the erythrocytes with the virus at 4°. The specificity of the RGA was controlled by the RZGA with immune serum /8/. /RZGA - hemagglutination inhibition reaction/.

For setting up hemadsorption we used a somewhat modified method of Buckley /6/. We poured off the medium from the test tubes with the infected cultures and the layer of cells was washed twice with a borate buffer (pH 9.0). After this the test tubes with the same buffer were incubated at 37° for 30 minutes. Then to the cultures we added an 0.25% suspension of goose erythrocytes in a phosphate buffer with a pH which was optimum for the RGA. The test tubes were left at room temperature for 45--60 minutes and the results were considered under the weak magnification of a microscope.

The RSK /complement fixation reaction/ was set up in the cold with 3 doses of complement in the modification of A. A. Smorodintsev. We used immune rabbit serum with a titer in the RTGA of 1:640. /RTGA - hemagglutination inhibition reaction/.

The morphological changes of the culture were studied after staining of the infected cells with hematoxylin-eosin.

Results

Hemagglutinating activity of VEE. In preliminary tests the optimum conditions were determined for exposing VEE hemagglutinins during the cultivation of the virus in tissues.

It was established that the highest titers of hemagglutinins are observed at a pH of 5.8--6.0. The use of a pH which is higher or lower than this leads to a sharp drop in the titers. The RGA goes similarly well both at 4° and at room temperature. The clearest results are obtained when using a 0.25% suspension of goose erythrocytes.

In the process of passaging VEE on a culture of chick embryo fibroblasts a gradual drop in hemagglutinating activity was noted right up to the complete disappearance of hemagglutinins by the 10--12th passage. This phenomenon was not connected with a decrease in the infecting ability of the virus, which following passaging remained at its previous level or even exceeded it.

Hemadsorption ability of VEE. In 6 hours following infection it is possible to note the appearance of hemadsorption, which is initially expressed

weakly and has a diffuse nature (1--2 erythrocytes are attached to each cell). Subsequently the results of the reaction become clearer, the number of erythrocytes adsorbed by the cells is increased noticeably, and in individual sectors of the layer aggregations of erythrocytes appear. By the 12th hour after the onset of infection, hemadsorption acquires a predominantly island nature. The following degeneration of the culture was accompanied by a gradual drop in the ability of the infected cells to adsorb erythrocytes.

The specificity of hemadsorption was checked with the help of the reaction of inhibition of hemadsorption with immune serum, which differed from the usual RZGA only by the fact that the serum was preliminarily treated with kaolin and goose erythrocytes /8/. It must be pointed out that it is possible to successfully use this reaction for the diagnosis of VEE and the titration of immune sera /6, 23, 24/.

Cytopathological effect of VEE. In 12--15 hours following infection the appearance is noted of individual spherical granulated cells which are scattered throughout the entire monolayer among the intact culture. The nuclei of affection of the cells are pycnotic, and in their cytoplasm foci of affection are observed along with vacuolization and intensification of basophilia. The nuclear membrane is outlined poorly and in places it cannot be determined at all. Subsequently a shrinking of the cells takes place, accompanied by the discharging of the layer, and a mass of formless detritus is formed (figure 1).

Multiplication of VEE in a culture of chick embryo fibroblasts. It is possible to visualize the nature of multiplication of VEE based on the change of titers of the infecting ability, hemagglutinins and the complement fixing antigen following a study of these indices during the infection process.

The results of a series of tests, which are presented in the table, visually demonstrate the bond between the accumulation of VEE and the degeneration of the culture which is taking place at the same time.

The first specific sign of the liberation of VEE is an increase in the quantity of infecting virus, which can be detected already in 4--5 hours following infection. By 6 hours the titer of the virus exceeds the specific "threshold of infectivity" and the virus hemagglutinin begins to become apparent in the cultural fluid. From this time the hemadsorption reaction becomes positive. Subsequently a rapid build up of the stated signs is noted. This testifies to the active multiplication of the virus. They reach their maximum expression by 18--24 hours, remaining at this level for the next 3 days.

The destruction of the cells of the culture sets in considerably later than the manifestation of the virus. Only after 12--15 hours following infection individual degenerate cells appear. From this moment the number of these begins to increase gradually. In 24 hours following infection hardly any intact cells remain in the culture, and after 48 hours the entire culture has perished. The described sequence of multiplication of VEE is also observed following the

infection of HeLa and SOTs cells by this virus.

This relationship between infectivity and hemagglutinating activity expresses graphically, and easily brings out the parallelism which exists between these two characteristics of the virus (figure 2). At the same time, the complement fixing antigen is determined in the cultural liquid later than the infecting virus and hemagglutinin.

Discussion

In contrast to the viruses of poliomyelitis, Herpes, smallpox vaccine and bacteriophages, which possess an explosion like type of multiplication with a simultaneous discharge from the cells /9, 15/, VEE, as well as other representatives of the arboviruses and certain myxoviruses, is characterized by the continuous prolonged liberation of virus particles into the surrounding medium due to their aging /2, 17, 22/.

Mussgay and Weibel /20/ established that each VEE infected cell eliminated up to 2700 plaque forming units of virus, and the period of discharge of the mature virus is only 20 seconds all told. This leads to a constant predominance of free virus over intracellular and makes it possible, based on the concentration of virus in the cultural fluid, to judge concerning the dynamics of its multiplication.

The cycle of multiplication of VEE may be divided into four successive phases: 1) the latent period, comprising 3--4 hours, when in the medium it is possible to detect an insignificant amount of residual virus, the titer of which hardly changes; 2) the period of exponential growth, lasting 12--15 hours and in nature reminiscent of the logarithmic phase of growth in bacteria; 3) the stationary period, lasting around 80 hours, when infectivity remains at the maximum level attained; here the thermal inactivation of the virus, which is subordinate to the kinetics of reactions of the first order /10, 14, 21/, levels due to the constant exit into the medium of the newly formed progeny; these contrasting processes create the position of an unique equilibrium, which is disrupted only after the inactivation of the virus begins to prevail over its multiplication (see figure 2); 4) the multiplication cycle of VEE is concluded with the period of death. On the whole the nature of accumulation of VEE is close to the classical curve of growth of a bacterial population /3/, and only the deficiencies in the methods for the quantitative calculation of virus, apparently, do not make it possible to determine the intermediate phases of acceleration and slowing down.

In 24--48 hours following infection the total death of the infected cells is noted. However, this is not accompanied by a decrease in the discharge of virus, the titer of which still remains at a high level for a long time. This paradoxical phenomenon requires further study. The continuing multiplication of VEE in the perished culture was established earlier by Hardy and Brown /14/, who showed that following several complete changes of the medium, during the course of the following 4 hours the titer of the virus again reaches a high

level each time.

Hemagglutinins of VEE are exposed only after a specific "threshold of infectivity" is achieved, and this equals 4.6--5.75 lg TCD_{50} (or $1.3 \cdot 10^6$ -- $2.6 \cdot 10^6$ plaque forming units). It is interesting to point out that the value of the "threshold of infectivity" is not the same in different viruses. For example, for the yellow fever virus it equals from -4.5 up to -5.0 lg LD_{50} /12/, for the virus of Japanese encephalytis -5.0 lg LD_{50} /23/, for the Semliki virus from -7.5 up to 8.0 lg LD_{50}, for the myxoviruses from -3.5 up to -3.9 lg LD_{50}, etc.

The existence of a parallelism between infectivity and the hemagglutinating activity (see figure 2) makes it possible to recommend the RGA as a simple and rapid test for determining the dynamics of multiplication of VEE in tissue culture. Here it can be easily calculated to which quantity of virus the titer of hemagglutinins found corresponds. As an early sign of the multiplication of VEE it is also possible to use the reaction of hemadsorption, which becomes positive long before the appearance of a cytopathological effect.

Attention is drawn to the fact of the gradual disappearance of the hemagglutinating activity of VEE during the process of passaging it on a culture of chick embryo fibroblasts. A similar phenomenon was detected in respect to the viruses of Japanese encephalytis, vaccine /7/, ECHO /19/, and Coxsackie /16/. The nature of this is unclear; it may be caused by selective variability or phenotypical mutation.

The later appearance in the cultural fluid of the complement fixing antigen may be explained, in an analogy with the data obtained in respect to the virus of tick-borne encephalytis, by the presence of a soluble complement fixing antigen which is not bound with the virus particle and passes from the cells into the surrounding medium more slowly than the infecting virus.

Conclusions

1. The virus of Venezuelan equine encephalomyelitis is capable of agglutinating goose erythrocytes at a pH of 5.8--6.0. The "threshold of infectivity" which is necessary for the exposure of VEE hemagglutinins comprises 4.6--5.7 lg TCD_{50} (~~1.3 $\cdot 10^6$ -- 2.6 $\cdot 10^6$~~ 1,300,000 -- 2,600,000 plaque forming units).

2. The reaction of hemadsorption makes it possible to expose the multiplication of VEE in 6--9 hours following infection of a sensitive culture.

3. The RGA and the reaction of hemadsorption may be used for determining the dynamics of multiplication of VEE and the early diagnosis of this virus.

4. A prolonged liberation of virus particles into the surrounding medium is characteristic for VEE.

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Figure 1. Chick embryo fibroblasts after 24 hours following infection with VEE. Stained with hematoxylin-eosin. X 400.

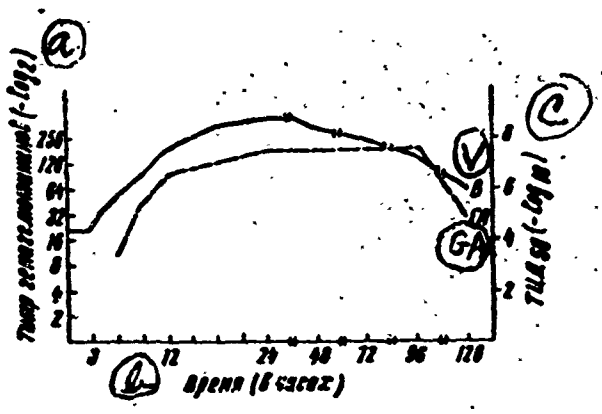

Figure 2. Change of infectivity (V) and hemagglutinins (GA) in the process of multiplication of VEE in a culture of chick embryo fibroblasts.
a - titer of hemagglutinins ($-\log_2$); b - time (in hours); c - TCD_{50} ($-\log_{10}$).

Connection between the cytopathological effect of VEE and the accumulation of infectivity, hemagglutinins and complement fixing antigen.

Time following infection (in hours)	1g CPD_{50}	titer of hemagglutinins	Hemadsorption	Complement fixing antigen	Cytopathological effect
1	4.3	0	0	-	-
2	4.5	-	-	-	-
3	4.5	0	0	0	0
4	5.0	0	-	-	-
6	5.75	8-16	+	0	0
9	6.6	32-64	+++	0	-
12	7.6	64-128	+++	H	+
18	8.5	128	+++	-	++
24	8.8	128-256	+++	H	+++
48	8.4	128-256	++	8	+++
72	7.9	128-256	+	2	+++
96	7.3	128-256	-	2	-
120	6.0	16-64	-	-	-
144	-	8-32	-	-	-

Legend: +, +++, degree of intensity of reaction; H, positive reaction with undiluted cultural fluid; -, test not set up; 0, negative result.